J Chem Ecol (2010) 36:432–440
DOI 10.1007/s10886-010-9775-5

Variation in and Responses to Brood Pheromone of the Honey Bee (*APIS mellifera* L.)

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Received: 12 November 2009 / Revised: 25 February 2010 / Accepted: 8 March 2010 / Published online: 31 March 2010

Abstract The 10 fatty acid ester components of brood pheromone were extracted from larvae of different populations of USA and South African honey bees and subjected to gas chromatography-mass spectrometry quantitative analysis. Extractable amounts of brood pheromone were not significantly different by larval population; however, differences in the proportions of components enabled us to classify larval population of 77% of samples correctly by discriminant analysis. Honeybee releaser and primer pheromone responses to USA,Africanized and–European pheromone blends were tested. Texas-Africanized and Georgia-European colonies responded with a significantly greater ratio of returning pollen foragers when treated with a blend from the same population than from a different population. There was a significant interaction of pheromone blend by adult population source among Georgia-European bees for modulation of sucrose response threshold, a primer response. Brood pheromone blend variation interacted with population for pollen foraging response of colonies, suggesting a self recognition cue for this pheromone releaser behavior. An interaction of pheromone blend and population for priming sucrose response thresholds among workers within the first week of adult life suggested a more complex interplay of genotype, ontogeny, and pheromone blend.

Key Words Brood pheromone variation · Honey bee · Pollen foraging · Sucrose response threshold · Africanized honey bees · African honey bees

Abbreviations
BP brood pheromone

Introduction

Honey bees, *Apis mellifera* L., are distributed naturally throughout Africa, Asia, Europe, and the Middle East (Ruttner 1988). European honey bees in the US are a mixture of approximately seven subspecies introduced by English and Spanish settlers in the 1600s and by bee breeders in the late nineteenth and early twentieth centuries (Sheppard 1989). The gene pool reflects these introductions in both the commercial and feral populations (Schiff et al. 1994; Schiff and Sheppard 1995). In 1956, a South African sub-species, *A. m. scutellata*, was introduced to Brazil, and queens from this sub-species mated with European males producing the Africanized honey bee, a genetic admixture of *A. m. scutellata* and European honey bees (Pinto et al. 2005). The first Africanized colony was discovered in the USA in 1990 (Pinto et al. 2005). As a consequence, there are two behaviorally different types of honey bees found in the Americas, referred to as Africanized and European honey bees.

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The comparative study of differences in pollen foraging behavior of these two types (Winston 1987) can help us understand the proximal mechanisms that regulate foraging activity. When fostered together in the same European colony, Africanized bees are more likely to forage for pollen and water than European bees (Danka et al. 1987; Pesante et al. 1987; Fewell and Bertram 2002; Pankiw 2003).

Along with other factors (reviewed in Page and Erber 2002), a mixture of larval chemicals, called brood pheromone (BP), influences various pheromone releaser responses in honey bee colonies, including the proportion of pollen foragers, pollen load weight, and pollen foraging trip frequency (Pankiw and Page 2001; Pankiw 2004a, 2007; Sagili 2007). Brood pheromone also elicits primer responses (Pankiw 2004b) such as, for example, affecting the age of first foraging; bees in colonies treated with a relatively low amount of BP forage at significantly younger ages than bees in colonies treated with a relatively high amount of BP (Le Conte et al. 2001; Sagili 2007).

Africanized bees forage at younger ages than European bees in common colony-rearing studies (Pankiw 2003). First foraging at a younger age and a greater probability to forage for pollen is associated with a greater sensitivity to sucrose (Page and Erber 2002; Pankiw et al. 2002; Pankiw 2003). Africanized bees have significantly lower response thresholds compared to European bees, when newly emerged or prior to any feeding experience (Pankiw 2003). Sucrose-response thresholds are modulated by BP, such that a relatively low amount of BP increases sucrose sensitivity and a relatively high amount decreases sucrose sensitivity (Pankiw and Page 2001; Pankiw 2004b; Pankiw et al. 2004). Treatment with a low dose of BP for 30 days increases the amount of brood area reared by colonies vs. controls, probably as a result of the combined suite of primer and releaser effects outlined above (Pankiw et al. 2004; 2008b; Sagili 2007).

Differences in a suite of pollen foraging behaviors between European and Africanized honey bees results in the Africanized bees collecting more pollen, rearing more brood, and their colonies reproducing more frequently in a tropical and subtropical environment (Winston 1992). This has led to the domination of the Africanized mitotype in Texas and much of the southern USA (Pinto et al. 2005; USDA-ARS 2008). Given that BP influences this suite of pollen foraging behaviors, a possible mechanism regulating differential pollen foraging between Africanized and European is variation in BP produced by the respective larvae, or differential response to BP by the respective adults.

Brood pheromone is composed of a blend of the methyl and ethyl esters of palmitic (16:0), stearic (18:0), oleic (18:1 Δ^{9}), linoleic (18:2 $\Delta^{9,12}$), and linolenic (18:3 $\Delta^{9,12,15}$) acids; the pheromone blend and extractable amounts vary with larval age and caste (reviewed in Pankiw 2004b and references therein). To date, all published studies on the effects of synthetic BP have been performed using the blend characterized from larvae of France (Le Conte et al. 1994; Pankiw 2004b), with most studies using colonies of European or mixed European lineage in the USA (Le Conte et al. 2001; Pankiw and Page 2001; Pankiw 2004a, b, 2007; Pankiw et al. 2004, 2008b). To date, there have been no published studies on variation in blend or amount of BP among different populations of same-stage larvae. Furthermore, the consequent behavioral or physiological effects of different blends of BP have been explored only superficially by using whole hexane extracts of larvae (Pankiw and Rubink 2002) or by not separating the effects of blend and dose (Pankiw and Page 2001).

The goal of this study was to examine BP blend variation and behavioral responses to any variation in three populations of bees characterized by geographical location and mitochondrial lineage: honey bees having European mitochondrial DNA from Texas and Georgia, USA, and bees having African mitochondrial DNA from Texas, USA and Pretoria, South Africa. We characterized BP blends by chromatography-mass spectrometry (GC-MS), and we examined differences in blend by using a discriminant analysis model. Because releaser and primer responses are modulated by different physiological systems (Pankiw 2004b), the effects of BP blends were cross-tested among the different populations of bees in a pollen foraging bioassay (releaser response) and a sucrose response threshold bioassay (a primer response).

Methods and Materials

Bee/Larval Sources Four bee populations were used. European queens and package bees were purchased from a breeder in Moultrie, Georgia, USA where no record of the African mitotype has been reported (USDA-ARS 2008). Africanized colonies were collected from Mission, Texas, USA. Texas-European honey bees were from Navasota, Texas, USA and had European mitochondrial DNA. The prevalence of Africanized bees in Texas suggested that introgression of African nuclear DNA in colonies with European mitochondrial DNA was likely (Pinto et al. 2005), meaning that this population may be intermediate. European and African mitochondrial lineage was confirmed by the method of Pinto et al. (2003). Texas-Africanized, Texas-European, and Georgia-European colonies were all maintained in a College Station, Texas apiary (30° 6′ N; 96° 32′ W). African (*A. m scutellata*) colonies were maintained in an apiary at the University of Pretoria, Pretoria, South Africa (25° 45’ S; 28° 14’ E). All the queens in this study were unrelated to each other and naturally mated.

Collection of Larvae for Chemical Analysis Larvae were collected from 8 Texas-Africanized, 8 Georgia-European colonies, 11 Texas-European colonies, and 7 South African colonies. Single frames containing larvae were removed from the colonies and brought within 30 min. to the laboratory, where larvae were removed from the cells with a gentle stream of water and collected into 1 mm mesh cloth. Larvae were lightly shaken dry and 10 fifth instars selected by weight and morphology (Rembold et al. 1980; Michelette and Soares 1993). Larvae were pooled in a glass beaker and soaked for 1 min. in 2 ml of 95% *n*-hexane containing 1 μg methyl myristate as an internal standard and 0.05% (w/w) *t*-butyl hydroquinone as an antioxidant. Addition of this antioxidant has been found to prevent significant chemical change of a mixture of fatty acid esters stored at room temperature for 72 wk (Pankiw et al. 2008a). Extracts were filtered through a Buchner funnel to remove particulate matter, and the larvae and glassware were washed further with 2 ml of 95% *n*-hexane, which was added to the extract. Extracts were stored in 7-ml glass, screw-top vials, sealed with aluminum foil and paraffin, and stored for up to 2 wk at -20°C before further processing. South African extracts were shipped by air from Pretoria to College Station, but in other respects were treated identically.

Extracts were concentrated to 1 ml by using a nitrogen stream in a water bath at 55°C and vortexed for 30 sec to wash down any chemicals on the glass. Extracts were fractionated by liquid chromatography using a column of 0.5 g of silica gel (70–230 mesh/60 Å; Sigma-Aldrich, St. Louis, MO, USA) in a Pasteur pipette plugged with a piece of dust-free paper cloth (Kimberly-Clark, Neenah, WI, USA). Before use, columns were rinsed with 10 ml each of 99% dichloromethane and 95% *n*-hexane. Extracts were placed on freshly prepared columns, and two fractions were eluted: the first, containing long and short-chain hydrocarbons, but no esters, with 10 ml of 95% *n*-hexane, and the second, containing the esters, with 10 ml of 99% dichloromethane. The dichloromethane fraction was evaporated to apparent dryness using a nitrogen stream in a water bath at 50°C, and then immediately reconstituted in 1 ml of 95% *n*-hexane and vortexed for 30 sec. This was concentrated to ca 100 μl and transferred to an autosampler vial containing a 250-μl glass insert; the vial previously containing the fraction was rinsed 3 times with 50 μl hexane and the rinse added to the insert. Solvent was removed from the fraction by heating to 50°C, after which the vial was cooled and 20 μl hexane containing 2 μg of n-octadecane (Sigma-Aldrich, St. Louis, MO, USA) added to the fraction as a reference. Fractionated extracts were stored at -20°C for up to 7 d until analysis by gas chromatography, using flame ionization or mass spectrometric detection.

Gas Chromatography Extracts were analyzed with a Hewlett Packard 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with splitless injection, flame ionization detection, a 60 m×0.25 mm ID HP-88 ([88%-cyanopropyl]-methylarylpolysiloxane, Agilent, Santa Clara, CA, USA), column. The inlet temperature was held at 60°C for 0.10 min and then increased to 250°C at 500°C min^{-1}. The column oven temperature was held at 50°C for 2 min, then increased to 170°C at 20°C min^{-}, held for 3 min, then increased to 230°C at 30°C min^{-1}. The carrier gas was hydrogen at a constant flow of 2 ml min^{-1}. Individual fatty acid ester retention times were identified by comparison with >99% pure standards (Sigma-Aldrich, St. Louis, MO, USA). For quantification, standard curves of peak area for each fatty acid ester were constructed. Extract ester quantities were corrected for methodological error by reference to the methyl myristate internal standard.

GC-MS GC-MS analyses were performed on a 6890 GC coupled with a 5975C Inert XL quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA). The GC was equipped with a 30 m×0.25 mm HP- 5MS [(5%-phenyl)-methylpolysiloxane], fused silica capillary column (Agilent, Santa Clara, CA, USA), and a splitless injector using helium as carrier gas at a constant flow of 1.0 ml min^{-1} and an inlet temperature of 300°C. The column temperature was programmed from 150° to 320°C at 20°C min^{-1}. The MS was operated in electron ionization mode (EI) at 70-eV electron energy, using selected ion monitoring (SIM) mode for the following *m/z* (indicative of): 74, 242 (methyl myristate), 74, 270 (methyl palmitate), 88, 284 (ethyl palmitate, 81, 294 (methyl linoleate), 84, 264 (methyl oleate), 79, 292 (methyl linolenate), 74, 298 (methyl stearate), 81, 308 (ethyl linoleate), 98, 310 (ethyl oleate), 79, 306 (ethyl linolenate), 88, 312 (ethyl stearate), and 85, 254 (octadecane).

Comparison of Total Amount of Brood Pheromone Extractable from Larvae of Four Different Populations Quantitated amounts of fatty acid esters were summed to provide a total amount of BP for each sample. Total amounts were transformed to a proportion of mean larval weight (ng BP/g larvae) to account for the expected significant differences between the races ($F_{3,114}$=22.01; P<0.001; data not shown). Total amount of BP/gram of larvae was not normally distributed (Kolmogorov-Smirnov; Z=3.9; df=115; P< 0.001;SPSS 2007) and was therefore transformed by natural log. Total amount was tested by ANOVA for homogeneity of variance (Sokal and Rohlf 1995; SPSS 2007).

Comparison of Brood Pheromone Blends among Four Larval Populations Amount of individual esters was transformed to proportion of total BP, natural log-transformed

and classified by predictive discriminant analysis (Huberty 1994; SPSS 2007). The natural log-transformed ester proportions were normally distributed, meaning that the assumption of multivariate normality was likely met (Huberty 1994). Group covariance matrices were not homogenous as tested by Box's M (M=486.1; $F_{110,15764}$= 3.7, P<0.001; Huberty 1994; SPSS 2007). A linear model was applied despite the potential differences in covariance matrices among larval populations for two reasons: first, Box's M is known to be overly sensitive, and second, the ratio of number of groups (n_g=3) to total samples (p=88) was low (n_g/p=0.03) (as per Huberty 1994). Under these conditions and using external classification through the leave-one-out method, linear discriminant functions perform as well or better than their quadratic counterparts (Huberty 1994). A total of 34 blends from Texas-Africanized colonies, 31 blends from Georgia-European colonies, and 23 blends from South African colonies were analyzed; colonies were sub-sampled 3–6 times. Because colonies are comprised of several patrilines (Tarpy et al. 2004), it was reasonable to include colony-level subsamples to better represent the genetic diversity of the colony. The leave-one-out classification scheme was utilized for external validation, meaning that each data point was categorized based on classification functions generated from all other points (Huberty 1994). Having no *a priori* hypothesis for unequal probabilities of group membership, equal probabilities were used (Huberty 1994). One condition of the discriminant analysis is that members of a group be assigned conclusively to a single category (Huberty 1994). We did not consider this condition true in the case of the Texas-European population, because a possibility existed for Africanized nuclear DNA introgression (Pinto et al. 2005). Therefore, BP blends from the Texas-European larvae were not categorized by the analysis, and rather were classified as ungrouped samples.

Synthetic Pheromone Preparation The synthetic blends of BP derived from Texas-European, Texas-Africanized, and Georgia-European populations were formulated from mean ester percentages obtained using GC and GC/MS, while unpublished percentages of the French blend were personally communicated by Y. Le Conte (Table 1). Synthetic esters were measured by mass and added to an amber glass vial (Grace-Davison, Columbia, MD, USA) with 0.05% of t-butyl hydroquinone (Sigma-Aldrich, St. Louis, MO, USA) added as an antioxidant. The synthetic BP blends were diluted in 95% n-hexane to a concentration of 0.5 μg/μl for application in the bioassays described below.

Pollen Foraging Response of Texas-European Colonies to France and Texas-European Derived Brood Pheromone Blends The pollen foraging bioassay was performed in 12 similarly-sized, Texas-European colonies, installed 1 wk earlier. Each colony was comprised of approximately 1 kg of bees, 1 queen, and few very young larvae (< 2 cm^2). Colonies received 0.6 mg of France or Texas-European derived BP characterized as above or an equal volume of solvent as blank control. This dose was selected through a series of dose-response trials performed prior to experimentation (as per Pankiw and Page 2001). Pheromone was applied on glass plates measuring 14×7 cm. Solvent was allowed to evaporate prior to placement in colonies. A single plate then was hung on metal wire between two frames in the middle of the colony. One hour after placement of glass plates the number of pollen and non-pollen foragers entering colonies was counted for a 5 min period (as per Pankiw et al. 1998). Pollen foragers are distinguishable visually from non-pollen foragers by the pollen pellets on their hind legs. Counts were performed between 08:00–10:00 h. All colonies received all treatments on subsequent days in a random, non-repeating manner. Counts were analyzed by 3×2 *chi-square* contingency table analysis for the effect of pheromone treatment on pollen to non-pollen forager ratio (Sokal and Rohlf 1995; SPSS 2007). Pairwise comparisons were performed using 2x2 *chi-square* contingency table analysis with the Dunn-Šidák method of correcting for possible Type I errors (Sokal and Rohlf 1995).

Pollen Foraging Responses to Texas-Africanized and Georgia-European Derived Brood Pheromone Blends by Texas-Africanized and Georgia-European Colonies The pollen foraging bioassay was performed as described above with 6 Texas-Africanized and 6 Georgia-European colonies each treated with 1.0 mg of the synthetic blend of Texas-Africanized or Georgia-European BP, or an equal volume of solvent control. Texas-Africanized and Georgia-European colony responses to pheromone treatments were statistically analyzed separately.

Modulation of Sucrose Response Thresholds by Texas-Africanized and Georgia-European Derived Brood Pheromone Bblends Frames of pupae collected from 6 Texas-Africanized and 8 Georgia-European colonies were taken to the lab and adults were allowed to emerge for 24 h in an incubator at 30°C, 55% RH. Three plexiglass/wire-mesh cages (1,400 cm^3) each received 300 of either Texas-Africanized or Georgia-European newly emerged bees. Cages were provisioned with 30 ml of 30% sucrose solution and 30 ml of water. Pheromone was applied on 7×7 cm glass plates hung by metal wire in the center of each cage. Each cage received one of three treatments: 1.8 mg of either Texas-Africanized or Georgia-European derived BP (Table 1), or an equivalent volume of solvent control. Solvent was allowed to evaporate prior to inserting

Table 1 Blend formulations of synthetic brood pheromone (%)

Fatty acid ester	European mtDNA			African mtDNA	
	France	Texas, USA	Georgia, USA	Texas, USA	South Africa
Methyl palmitate	3.0	6.9	2.9	4.1	6.0
Ethyl palmitate	3.0	3.9	3.9	4.7	8.8
Methyl stearate	17.0	15.6	6.5	14.9	25.6
Ethyl stearate	7.0	6.4	8.1	10.3	14.5
Methyl oleate	25.0	16.8	20.6	9.0	11.5
Ethyl oleate	8.0	15.4	13.8	15.9	12.9
Methyl linoleate	2.0	2.9	4.2	9.3	4.5
Ethyl linoleate	1.0	5.4	12.1	6.4	4.9
Methyl linolenate	21.0	13.8	16.9	11.7	2.8
Ethyl linolenate	13.0	12.9	11.0	13.7	8.5

glass plates into cages. Cages were stored in a dark incubator at 30°C, 55% RH. Treatment plates, sucrose, and water were changed daily. Bees were reared thus for 5 d so that they were tested within the first week of adult life (as per Pankiw and Page 2001). Mortality over the course of treatment was less than 15 bees per cage. On the sixth day, a subset of 60 bees was collected from each cage and their sucrose response thresholds were measured using the proboscis extension reflex assay.

Proboscis Extension Reflex Bioassay Honey bees reflexively respond by extending the proboscis when a sufficiently concentrated solution of sucrose is applied to the antennae (Pankiw and Page 2001). The response threshold of an individual may be estimated by the lowest concentration that elicits proboscis extension when bees are presented with an ascending concentration series (Pankiw and Page 2001; Pankiw and Rubink 2002; Pankiw et al. 2002; Pankiw 2003). The proboscis extension reflex bioassay followed previously reported methodologies (Pankiw and Page 2001; Pankiw et al. 2004). Briefly, bees were restrained by thin strips of tape such that their heads and mouthparts moved freely. Bees were allowed 1 h to acclimate to restraint before testing. All bees were tested for proboscis extension to antennal stimulation with water; those responding were allowed to drink water to satiation. Bees then were presented with a logarithmically ascending series of sucrose concentrations sucrose (0.1, 0.3, 1.0, 3.0, 10, and 30%). A single droplet of sucrose concentration was touched to each antenna for <3 sec; ascending concentrations were applied at least 5 min apart. A positive response was recorded if the bee extended her proboscis. Positive responses to sucrose were summed to yield scores that were analyzed by Kruskal-Wallis (Sokal and Rohlf 1995; SPSS 2007). A high score corresponded to high sensitivity or a low response threshold to sucrose and a low score to low sensitivity or a high response threshold to sucrose. Mann-Whitney *U* was performed to assess pairwise differences in sucrose response threshold by treatment using the Dunn-Šidák method as above (Sokal and Rohlf 1995; SPSS 2007).

Results

Comparison of Total Amount of Brood Pheromone Extractable from Larvae of Four Different Populations Total amount of BP extractable from the larval cuticle did not differ among the four honey bee populations ($F_{3,114}$=0.9; P=0.45; SPSS 2007). Mean total extractable amounts were 662±166 ng pheromone/g larvae.

Comparison of Brood Pheromone Blends among Four Larval Populations Two discriminant functions were generated. Means of the functions were different among populations (χ^2=117.5, 20 *df*, P<0.001; SPSS 2007). Squared canonical correlation coefficients showed that function 1 explained 57.2% of the variation among the groups, while function 2 explained 37.2%. Overall, 77% of the samples were correctly classified. That is, 66.7% of the Georgia-European blends categorized correctly, 30% of this group were categorized as Texas-Africanized, and 3.3% as South African. Among the Texas-Africanized blends, 76.5% were correctly categorized, 11.8% were categorized as Georgia-European, and 11.8% as South African. The categorization of South African blends was 91.3% correct, while 4.3% were categorized as Georgia-European and 4.3% as Texas-Africanized. Of the 18 Texas-European blends, 39% were classified as Georgia-European, 33% as Texas-Africanized, and 28% as South African (Fig. 1). Function 1 was the primary discriminator between Texas-Africanized and Georgia-European blends and the South African blends, while function 2 was the primary discriminator

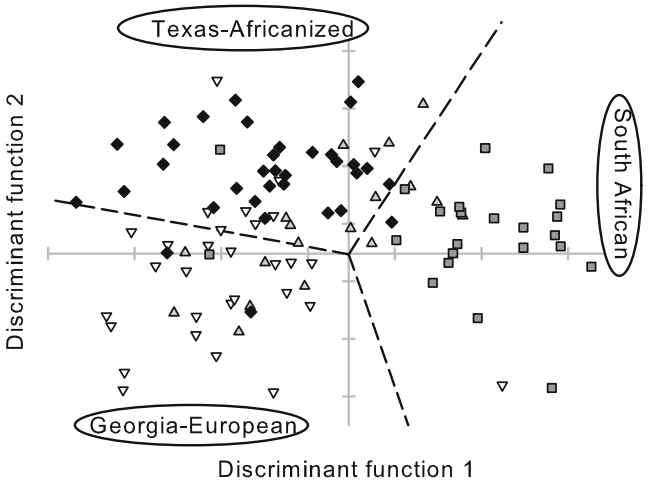

Fig. 1 Scatterplot of the discriminant scores for brood pheromone ester proportions extracted from larvae of colonies of Texas-Africanized and Georgia-European honey bees and South African. Each point represents a single sub-sample. Circled labels indicate the classification of all sub-samples within the territories demarcated by the dashed lines. Georgia-European samples are represented by open, upside-down triangles, Texas-European by grey triangles, Texas-Africanized by black diamonds, and South African samples are represented by dark grey squares

between the Georgia-European and Texas-Africanized blends (Fig. 1).

Plotting the structure matrix coefficients of the transformed ester proportions to the discriminant functions revealed the relative importance of each ester to the classification of the blends (Fig. 2). Vectors were plotted at an angle, the tangent of which was the ratio of function 2 to function 1. The method of calculating the magnitude of the vectors was adapted from Overall and Klett (1972) as the ratio of the standard error of the group means for each ester proportion to the mean standard error of the ester proportions within each group. The ethyl and methyl esters of palmitic (16:0) and stearic (18:0) acids were highly positively correlated to function 1, while methyl linolenate (Me-18:3$\Delta^{9,12,15}$ was highly negatively correlated (Fig. 2). Palmitic and stearic acids are both saturated, while linolenic acid is triple-unsaturated. This means that the South African blends were more likely to exhibit a high proportion of unsaturated fatty acid esters and a relatively low proportion of poly-unsaturated fatty acid esters, particularly methyl linolenate. Both methyl and ethyl linolenate were highly positively correlated with function 2, while methyl oleate (Me-18:1Δ^{9} and ethyl linoleate (Et-18:2 $\Delta^{9,12}$ were highly negatively correlated. Georgia-European blends were more likely to exhibit a higher proportion of methyl oleate and ethyl linoleate, and a lower proportion of the esters of linolenic acid.

Pollen Foraging Response of Texas-European Colonies to France and Texas-European Derived Brood Pheromone Blends Proportion of pollen to non-pollen foragers differed by pheromone treatment (χ^2=64.0, 2 *df*, P<0.001; Fig. 3a; SPSS 2007). Pairwise comparisons indicated that all treatments were different from each other, with the greatest ratio of pollen to non-pollen foragers in response to the Texas-European BP blend compared to the French blend (χ^2=15.1, 1 *df*, P<0.001) or the solvent control (χ^2=64.2, 1 *df*, P< 0.001). Pollen to non-pollen forager ratios in response to the French blend was greater than control (χ^2=22.1, 1 *df*, P< 0.001), which was consistent with previous studies (Pankiw et al. 1998; Pankiw and Page 2001; Pankiw 2004a).

Pollen Foraging Responses to Texas-Africanized and Georgia-European Derived Brood Pheromone Blends by Texas-Africanized and Georgia-European Colonies Proportions of pollen to non-pollen foragers also were different by pheromone treatment among Georgia-European colonies (χ^2=121.4, 2 *df*, P<0.001; Fig. 3b). Pollen to non-pollen forager ratios were greatest in response to treatment with the Georgia-European derived BP blend compared to either the Texas-Africanized blend (χ^2=3.3, 1 *df*, P=0.038) or solvent control (χ^2=109.1, 1 *df*, P<0.001). Pollen to non-pollen forager ratios in response to the Texas-Africanized blend was greater than control (χ^2=72.6, 1 *df*, P<0.001).

Proportions of pollen to non-pollen foragers differed by treatment among Texas-Africanized colonies (χ^2=59.8,

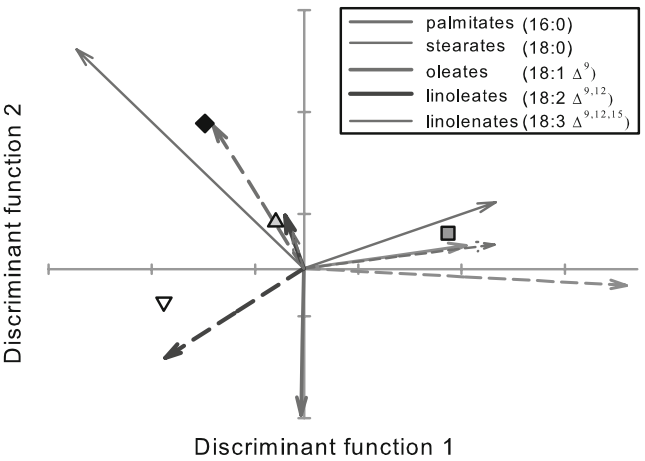

Fig. 2 Strength of the relationship of the proportions of brood pheromone esters to the discriminant functions. Solid lines represent methyl esters and dashed lines ethyl esters of palmitic, stearic, oleic, linoleic, and linolenic acids. Points represent the discriminant score of the group centroid: Georgia-European by an open, upside-down triangle, Texas-European by a grey triangle, Texas-Africanized by a black diamond, and South African by a dark grey square. Vector angles represent the ratio of the correlation of the ester proportion to the discriminant function and magnitudes represent the ratio of between- to within-groups standard error

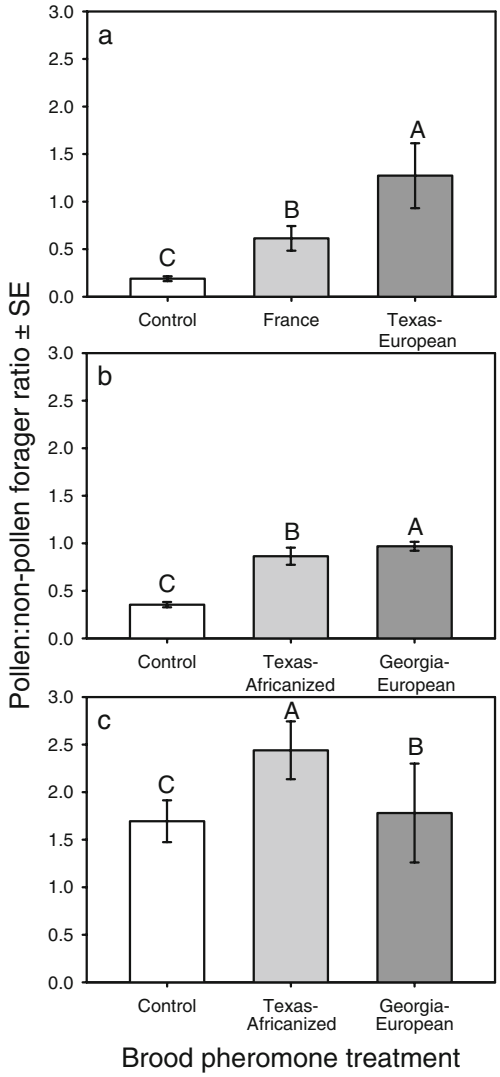

Fig. 3 Pollen to non-pollen forager ratio of **a** Texas-European colonies treated with French or Texas-European derived synthetic brood pheromone blends or solvent control, **b** Georgia-European honey bee colonies in response to Texas-Africanized and Georgia-European synthetic brood pheromone blends, and **c** Texas-Africanized honey bee colonies in response to Texas-Africanized and Georgia-European synthetic brood pheromone blends. Each letter denotes a statistically different subset (*Chi-square*; $P<0.017$)

2 *df*, $P<0.001$; Fig. 3c). Pollen to non-pollen forager ratios were highest in response to the Texas-Africanized blend when compared to the Georgia-European blend ($\chi^2=58.8$, 1 *df*, $P<0.001$) or solvent control ($\chi^2=26.0$, 1 *df*, $P<0.001$). Pollen to non-pollen forager ratios in response to the Georgia-European blend were greater than control ($\chi^2=7.1$, 1 *df*, $P=0.004$).

Modulation of Sucrose Response Thresholds by Texas-Africanized and Georgia-European Derived Brood Pheromone Georgia-European bees exhibited different proboscis extension reflex score distributions between pheromone treatments ($\chi^2=77.2$, 2 *df*, $P<0.001$: Fig. 4a). In Mann-Whitney comparisons, scores were higher when bees were treated with Texas-Africanized blend compared to control, but non-significantly ($Z=-2.3$, $P=0.023$). Scores were lower when bees were treated with Georgia-European blend when compared to control ($Z=-6.5$, $P<0.001$). Georgia-European bees exhibited higher scores when treated with Texas-Africanized derived BP blend compared to those treated with the Georgia-European blend ($Z=-8.3$, $P<0.001$). Proboscis extension reflex scores were not significantly different by pheromone treatment in Texas-Africanized bees ($\chi^2=1.4$, 2 *df*, $P=0.51$; Fig. 4b).

Discussion

Brood pheromone component proportions, blends, were significantly and sufficiently different to correctly classify the population of origin with 77% accuracy. The South African BP blends were characterized by higher proportion of esters of the saturated palmitic and stearic acids, both unsaturated fatty acids. Esters of unsaturated fatty acids oxidize more readily and are therefore more labile than saturated fatty acid esters (Kotz et al. 2006). It is reasonable to hypothesize that the South African blends may be more

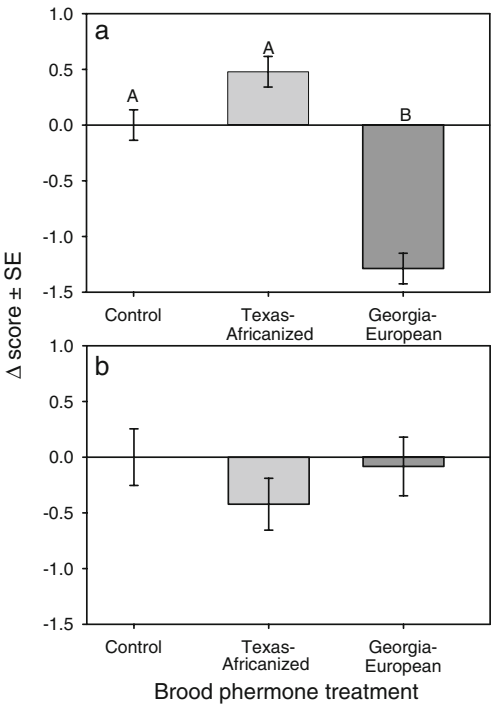

Fig. 4 Proboscis extension reflex scores of **a** Georgia-European or **b** Texas-Africanized honey bees in cages treated with Georgia-European or Texas-Africanized synthetic brood pheromone blends and compared as a mean difference from solvent control treated bees. Letters denote statistically different scores (Mann-Whitney *U*; $P<0.017$)

stable over time relative to the European blends and represent a more persistent pheromone cue. Proportions of methyl oleate were highest in the European blends compared to those extracted from populations with African maternal lineage and suspected Africanized DNA introgression. Methyl oleate has been demonstrated as an attractant for young adult bees in queen retinue bioassays (Keeling et al. 2003). Texas-Africanized blends were characterized by relatively higher proportions of ethyl oleate. Ethyl oleate increases the protein extractable from the hypopharyngeal glands after 14 days when fed to caged bees in the presence of an unmated queen (Mohammedi et al. 1996). Hypopharyngeal glands produce proteinaceous secretions of brood food fed to larvae (Winston 1987). Combined, these differences may be indicative of differential nursing behaviors among the different populations. Interestingly, the Texas BP blends from larvae with European *mt*DNA were evenly categorized among Texas-Africanized, Georgia-European, and South African categories, meaning that these blends may be viewed as intermediate. This result is consistent with our hypothesis of nuclear DNA introgression of African genes in this Texas-European population. Geographic isolation and subsequent selection of naturally and human distributed honey bee races are potential contributory factors that affect pheromone variation. Differential apicultural selection pressures on colonies for pollination or honey production in the USA, France, and South Africa, also may have changed BP blend. Further studies of BP blend variation among single patrilines of larvae will be necessary to determine the genetic and environmental components of this variation.

Contrary to our hypothesis, the Africanized blend did not release a greater proportion of pollen foragers in all colony types; instead, colonies responded with the greatest proportion of pollen foragers when treated with their own synthetic blend of pheromone. Thus, pollen foraging behavior cannot be explained through differences in extractable amounts or blend of BP. In contrast, Pankiw and Rubink (2002) found no race-by-pheromone interaction when testing pollen foraging response to whole hexane extracts of larvae. In our discriminant analysis, Texas-European blends of BP were intermediate between Georgia-European and Texas-Africanized blends. Because Pankiw and Rubink (2002) used Texas-European colonies, it is possible that the differences between Texas-Africanized and Texas-European BP blends were not sufficient to elicit a differential pollen foraging response. As shown previously, (Pankiw and Rubink 2002), Texas-Africanized colonies fielded a higher proportion of pollen foragers relative to Georgia-European colonies. Population differences in pheromone blend and response to blend may be the result of genetic isolation, however, BP blends varied within each population and even within colonies (personal observations). Considering that colonies consist of multiple patrilineal genotypes (Tarpy et al. 2004), forager genotype may interact with pheromone blend to act on response thresholds to BP that regulate division of foraging labor and contribute to intracolony variation in foraging ontogeny and specialization (*reviewed in* Winston 1987). Careful measures of intracolony variation in BP blend and responses of individuals to different blends will be necessary to explore this supposition further.

Modulation of sucrose response thresholds, a primer response, also showed a pheromone blend by population-dependent response interaction. The BP blend derived from Georgia-European colonies significantly decreased sucrose responsiveness in bees of the same population within the first week of adult life vs. the control response. However, Texas-Africanized bees appeared to be insensitive to the modulating effects of BP on sucrose sensitivity regardless of the BP blend environment in which they were reared. This study is the first to measure sucrose response threshold modulation by blends of BP other than the French blend and among individuals with African mitochondrial DNA. Only the Georgia-European blend modulated sucrose responsiveness among bees of USA origin that have a European mitotype in a manner predicted from previous studies (Pankiw and Page 2001; Pankiw et al. 2004). The highly responsive baseline of Africanized bees, consistent with that observed in previous studies (Pankiw and Rubink 2002), may be indicative of a more mature sucrose sensitivity status associated with a faster rate of foraging ontogeny (Pankiw 2003). If BP modulates sucrose responsiveness in pre-foraging Africanized bees, then we predict that modulation should be measurable in bees that are ontogenetically younger than those tested here, given that the French synthetic blend does not modulate USA European ontogenetically mature foragers (Metz and Pankiw unpublished). Brood pheromone modulated sucrose responsiveness is likely a complex interaction of baseline responsiveness, amount, and blend of BP.

This study has shown that BP blend changed among same-stage larvae from different populations. A pheromone comprised of at least 10 compounds presents an enormous challenge to our understanding of how honey bees process mixtures of pheromone compounds. The pattern observed for pollen foraging responsiveness to variation in blends of BP suggests BP may serve as a recognition cue. That is, in each case, there was a positive and significant response to a synthetic blend of BP that was characterized from a source of larvae from the same population as the colony. Pheromone modulated sucrose response threshold did not follow the same pattern, however, primer and releaser responses are not necessarily coordinated but rather uncoupled generating a complex interaction to variation in BP at various levels within a colony.

Acknowledgements For editorial notes and field assistance, the authors are indebted to Vincent Dietemann. The authors also thank Christian Pirk, GW Burlin, Art Cavazos, Lizette Peters, Ramesh Sagili, and Howard Williams for technical support and assistance. This study was funded by grants from the USDA-NRI 2004-35302-15031 and the Texas Honey Bee Legislative Initiative.

References

DANKA, R. G., HELLMICH, R. L., RINDERER, T. E., COLLINS, A. M. 1987. Diet-selection ecology of tropically and temperately adapted honey bees. *Anim. Behav.* 35:1858–1863.

FEWELL, J. H., and BERTRAM, S. M. 2002. Evidence for genetic variation in worker task performance by African and European honey bees. *Behav. Ecol. Sociobiol*. 52:318–325.

HUBERTY, C. J. 1994. Applied Discriminant Analysis. Wiley, New York.

KEELING, C. I., SLESSOR, K. N., HIGO, H. A., and WINSTON, M. L. 2003. New components of the honey bee (*Apis mellifera* L.) queen retinue pheromone. *Proc. Nat. Acad. Sci.USA.* 100:4486–4491.

KOTZ, J. C., TREICHEL, P. M., WEAVER, G. C. 2006. Chemistry and General Reactivity. Brooks, Belmont.

LE CONTE, Y., SRENG, L., and TROUILLER, J. 1994. The recognition of larvae by worker honey bees. *Naturwissenschaften* 81:462–465.

LE CONTE, Y., MOHAMMEDI, A., and ROBINSON, G. E. 2001. Primer effects of a brood pheromone on honeybee behavioural development. *Proc. R. Soc. London*, B 268:163–168.

MICHELETTE, E. R., and SOARES, A. E. E. 1993. Characterization of the preimaginal development stages in Africanized honey bee workers (*Apis mellifera* L). *Apidologie* 24: 431–440.

MOHAMMEDI, A., CRAUSER, D., PARIS, A., and LE CONTE, Y. 1996. Effect of a brood pheromone on honeybee hypopharyngeal glands. *C. R. Acad. Sci. Paris, Sciences de la vie.* 319:769–772.

OVERALL, J. E., and KLETT, C. J. 1972. Applied Multivariate Analysis. McGraw-Hill, New York.

PAGE, R. E., and ERBER, J. 2002. Levels of behavioral organization and the evolution of division of labor. *Naturwissenschaften* 89:91–106

PANKIW, T. 2003. Directional change in a suite of foraging behaviors in tropical and temperate evolved honey bees (*Apis mellifera* L.). *Behav. Ecol. Sociobiol*. 54:458–464.

PANKIW, T. 2004a. Brood pheromone regulates foraging activity of honey bees (Hymenoptera: Apidae). *J. Econ. Entomol*. 97:748–751.

PANKIW, T. 2004b. Cued in: honey bee pheromones as information flow and collective decision-making. *Apidologie* 35:217–226.

PANKIW, T. 2007. Brood pheromone modulation of pollen forager turnaround time in the honey bee (*Apis mellifera* L.). *J. Insect. Behav.* 20:173–180.

PANKIW, T., and PAGE, R. E. 2001. Brood pheromone modulates sucrose response thresholds in honeybees (*Apis mellifera*). *Behav. Ecol. Sociobiol.* 49:206–213.

PANKIW, T., and RUBINK, W. L. 2002. Pollen foraging response to brood pheromone by Africanized and European honey bees (*Apis mellifera* L.). *Ann. Entomol. Soc. Am*. 95:761–767.

PANKIW, T., PAGE, R. E., and FONDRK, M. K. 1998. Brood pheromone stimulates pollen foraging in honey bees (*Apis mellifera*). *Behav. Ecol. Sociobiol.* 44:193–198.

PANKIW, T., TARPY, D. R., and PAGE, R. E. 2002. Genotype and rearing environment affect honeybee perception and foraging behaviour. *Anim. Behav.* 64:663–672.

PANKIW, T., ROMAN, R., SAGILI, R. R., and ZHU-SALZMAN, K. 2004. Pheromone-modulated behavioral suites influence colony growth in the honey bee (*Apis mellifera*). *Naturwissenschaften* 91:575–578.

PANKIW, T., LAFONTAINE, J. P., and AVELINO, N. 2008a. Stabilized synthetic brood pheromone for manipulating the behaviour and physiology of honey bees. International Patent WO/2008/028269. Published March 13, 2008.

PANKIW, T., SAGILI, R. R., and METZ, B. N. 2008b. Brood pheromone effects on colony protein supplement consumption and growth in the honey bee (Hymenoptera: Apidae) in a subtropical climate. *J. Econ. Entomol*. 101:1749–1755.

PESANTE, D., RINDERER, T. E., and COLLINS, A. M. 1987. Differential pollen collection by Africanized and European honeybees in the neotropics. *J. Apic. Res*. 26:24–29.

PINTO, M. A., JOHNSTON, J. S., RUBINK, W. L., COULSON, R. N., and PATTON, J. C., SHEPPARD, W. S. 2003. Identification of Africanized honey bee (Hymenoptera: Apidae) mitochondrial DNA: validation of a rapid polymerase chain reaction-based assay. *Ann. Entomol. Soc. Am.* 96:679–684.

PINTO, M. A., PATTON, J. C., COULSON, R. N., and JOHNSTON, J. S. 2005. Africanization in the United States: replacement of feral European honeybees (*Apis mellifera* L.). *Genetics* 170:1653–1665.

REMBOLD, H., KREMER, J. P., and ULRICH, G. M. 1980. Characterization of postembryonic developmental stages of the female castes of the honey bee, *Apis mellifera* L. *Apidologie* 11: 29–38.

RUTTNER, F. 1988. Biogeography and Taxonomy of Honeybees. Springer-Verlag, Berlin.

SAGILI, R. R. 2007. Evaluation of physiological and pheromonal factors regulating honey bee, *Apis mellifera* L. (Hymenoptera: Apidae) foraging and colony growth. Dissertation, Texas A&M University.

SCHIFF, N. M., and SHEPPARD, W. S. 1995. Genetic analysis of commercial honey bees (Hymenoptera: Apidae) from the southern United States. *J. Econ. Entomol*. 88:1216–1220.

SCHIFF, N. M., SHEPPARD, W. S., LOPER, G. M., and SHIMANUKI, H. 1994. Genetic diversity of feral honey bee (Hymenoptera: Apidae) populations in the southern United States. *Ann. Entomol. Soc. Am*. 81:886–889.

SHEPPARD, W. S. 1989. A history of the introduction of honey bee races into the United States. *Am. Bee J.* 129:617–619 and 664–667.

SOKAL, R. R., and ROHLF, F. J. 1995. Biometry: The Principles and Practice of Statistics in Biological Research. Freeman, New York.

SPSS (2007) SPSS: Advanced Statistics 16.01 for Windows. SPSS, Inc., Cary, NC.

TARPY, D. R., NIELSEN, R., and NIELSEN, D. I. 2004. A scientific note on the revised estimates of effective paternity frequency in *Apis*. *Insectes Soc*. 51:203–204.

USDA-ARS. 2008. Spread of Africanized honey bees by year, by county. United States Department of Agriculture. http://www.ars.usda.gov/Research/docs.htm?docid=11059. Accessed 20 Feb 2009.

WINSTON, M. L. 1987. The Biology of the Honey Bee. Harvard University, Cambridge.

WINSTON, M. L. 1992. The biology and management of Africanized honey bees. *Annu. Rev. Entomol*. 37:173–193.